The Quantification of Glycosaminoglycans: A Comparison of HPLC, Carbazole, and Alcian Blue Methods

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Abstract: Glycosaminoglycans (GAGs) are linear polysaccharides that are found in the extracellular matrix and biological fluids of animals where they interact with hundreds of proteins and perform a variety of critical roles. There are five classes of animal GAGs: heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA). Many biological functions can be monitored directly by their impact on GAG quantity. Thus, simple, sensitive, and robust GAG quantification methods are needed for the development of biomarkers. We have systematically compared three available GAG quantification assays including an HPLC-based assay, a simplified Alcian Blue assay, and a miniaturized carbazole assay. The carbazole and Alcian Blue assays were reproducible and simple to perform in general lab settings, but had important limitations: The carbazole assay could not detect KS and it overestimated GAGs that were contaminated with salts or dissolved in PBS. The Alcian Blue assay detected only those GAGs that were sulfated. In contrast, while the HPLC method was time-consuming, it was a robust and sensitive assay that not only detected all GAGs but also quantified glucosamine-GAGs and galactosamine-GAGs simultaneously. The HPLC assay was not affected by salt or level of GAG sulfation and it yielded reproducible values for all types of GAGs tested. These results suggest that an automated HPLC assay would be generally useful for the routine measurement of a panel of GAG-based biomarkers while the carbazole assay and the Alcian Blue assays could prove valuable for more specific purposes.

INTRODUCTION

Glycosaminoglycans (GAGs) are linear polysaccharides comprised of repeating hexosamine-containing disaccharides that are sulfated to varying degrees. Most animal cells synthesize multiple types of GAGs including heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA). HS, CS, and DS are assembled on proteoglycan core proteins in the Golgi through linkage tetrasaccharides (GlcA-Gal-Gal-Xyl-Ser). KS is assembled on specific core proteins through N-linked (KSI) or O-linked (KSII) oligosaccharides. HA is unique in that it is unsulfated and is directly secreted without a core protein into the extracellular space.

HA and HS consist of repeating disaccharide units of GlcNAc and GlcA while KS is composed of alternating N-acetyl glucosamine (GlcNAc) and galactose. CSs consist of alternating GalNAc and GlcA/IdoA. They are classified as CS types A, B, C, D, and E according to the predominant disaccharide form (Table **1**). CSs isolated from animal tissues usually feature one major type of repeating disaccharide, but always contain minor species of other disaccharides. DS, also known as CS-B, is distinguished from other CS varieties by its high content of IdoA residues and its unique anticoagulant properties [1].

The most studied GAG, heparin, is a heavily sulfated heparan sulfate made by mast cells. Heparin is used clinically as an anticoagulant drug but also possesses anti-sepsis [2], anti-spontaneous abortion [3], anti-selectin mediated inflammation, anti-interstitial cystitis, and anti-tumor metastasis properties [4-8]. Commercial heparin is expressed as units/mg of anticoagulant activity because both its purity and quality are difficult to control. CS and glucosamine are also of clinical interest, ranking third among all top-selling nutriceuticals in the United States as a supplement for treating osteoarthritis [9].

Many biological functions can be monitored directly by their impact on GAGs since hundreds of proteins interact with GAGs [10]. GAGs are directly involved in many signaling pathways [11]. In addition, GAG biosynthesis is regulated by a variety of chemokines, cytokines, and growth factors [12,13]. Thus, simple, sensitive, and robust GAG quantification methods are needed for the development of biomarkers.

GAGs can be quantified and analyzed by NMR [14], MS [15], LC-MS [16], enzymatic post column fluorescence HPLC [17], electrophoretic methods [18], and by more general methods for monosaccharide composition analysis such as high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) [19]. However, a simple, direct, sensitive, and robust quantification method is needed for routine GAG quantification.

Three direct GAG quantification methods, including HPLC [20], carbazole [21,22], and Alcian Blue [23] assays have been established. However, a systematic comparison of these three assays in terms of sensitivity, reliability, and limitations has not been reported. We have systematically quantified CS-A, CS-B, CS-C, CS-D, CS-E, completely

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Table 1. Disaccharide Content of Various Chondroitin Sulfates

Major disaccharides	Other disaccharides found	
CS-A: GlcA-GalNAc4S	GlcA-GalNAc	IdoA2s-GalNAc
CS-B: IdoA2S-GalNAc4S	IdoA-GalNAc6S	GlcA3S-GalNAc
CS-C: GlcA-GalNAc6S	IdoA-GalNAc4S6S	GlcA3S-GalNAc4S
CS-D: GlcA2S-GalNAc6S	IdoA2S-GalNAc4S6S	GlcA3S-GalNAc4S6S
CS-E: GlcA-GalNAc4S6S	IdoA2S-GalNAc	GlcA3S-GalNAc6S

desulfated re-N-acetylated (CDSNAc)-heparin, N-desulfated re-N-acetylated (NDSNAc)-heparin, and heparin with our previously published HPLC assay [20], a simplified Alcian Blue assay [24], and a miniaturized carbazole assay. We examined the performance of each assay with sulfated and de-sulfated GAGs and in the presence of salt and DNA. The HPLC method detected and quantified all the GAGs we tested and was unaffected by salt or DNA contaminants. In addition, the HPLC method detected cross contaminations of certain glucosamine- and galactosamine-GAGs. The Alcian Blue assay could only detect sulfated GAGs. Contrary to previous reports, we found that the carbazole assay produced false positive signals due to the presence of salt. This information may prove useful in the design of simple GAG-based assays.

RESULTS

HPLC Analysis

GAG preparations are usually a mixture of GAG species due to their biological co-expression and structural similarities. Thus, whether GAGs are isolated, digested, fractionated, or purchased, their purity and concentration must be determined before they are used in biological assays. We previously developed an HPLC-based method that measures all GAGs tested with great precision and accuracy [20]. It can readily detect 10pmol of GlcN or GalN. Using our standard procedure, we analyzed commercially available CS A, B, C, D, E, and heparin, adding norleucine (NorL) to each GAG sample for normalization purposes. The reduced glucosamine (GlcN-OH), galactosamine (GalN-OH), and NorL profiles of 1µg dry weight-equivalents of different GAGs are shown in Fig. (**1**). The GlcN-OH peak was derived from the glucosamine-containing GAGs, including HS, KS, and HA, while the GalN-OH peak was from CS and DS. As expected, the heparin profile had no GalN-OH peak and the CS D and E profiles had no GlcN-OH peak. However, CS-A, CS-B, and CS-C were contaminated with glucosamine containing GAGs as evidenced by the GlcN-OH peaks in their profiles (Fig. **1**).

We also analyzed additional lots of heparin, CS-A, CS-B, and CS-C from the same source (Sigma) and CS-E from a different manufacturer (Fig. **2**). While this Sigma heparin preparation (Porcine mucosa heparin, catalog number H4784, lot 104K1177) had no detectable GalN-OH (Fig. **1**), an earlier lot of the same sigma reagent produced a significant GalN-OH peak (Fig. **2**). Moreover, the intensity of GlcN-OH peaks in CS-A, CS-B, and CS-C differed between lots. Only one CS-C lot produced a GlcN-OH peak (Fig. **1**). While no GlcN peaks were detected in squid cartilage CS-E from either commercial source, CS-E obtained from Seikagaku (Fig. **1**) yielded twice the detectable GAG per unit dry weight than CS-E obtained from Calbiochem (Fig. **2**). These observations demonstrated that the purity of commercial GAG preparations is highly variable.

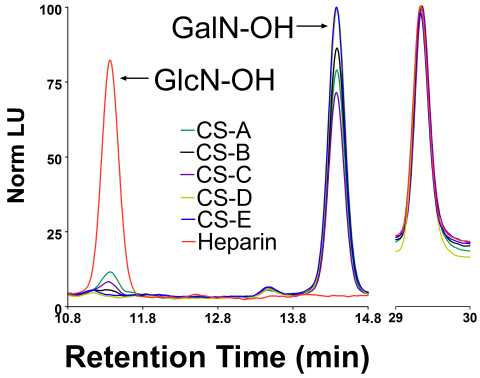

Fig. (1). CS-A, CS-B, CS-C, CS-D, CS-E, and heparin were converted to their respective monosaccharides (GlcN and GalN) via hydrolysis, reduced to GlcN-OH or GalN-OH by sodium borohydride treatment. The OPA derivatives were separated [20]. The elution positions and intensities for 1µg dry weight of GlcN-OH and GalN-OH, and the internal standard norleucine (NorL) are shown. The data is representative of three independent experiments.

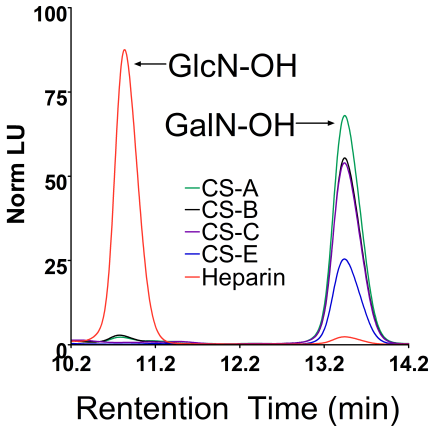

Fig. (2). GlcN-OH and GalN-OH elution profiles of different batches of CS-A, CS-B, CS-C, CS-E, and heparin. The degree of GAG-cross contamination for each GAG differed in a second batch (Fig. **1**). In a subsequent experiment, the same degree of contamination was observed for each GAG batch.

Treatment of HS with HNO_2 at pH 1.5 selectively degrades N-sulfated HS and has no effect on CS [25]. We used this reaction to determine whether the putative GlcN peaks derived from CS-B (Fig. **2**) were indeed derived from HS contamination. We therefore treated the CS-B preparation with HNO_2 at pH 1.5, removed any HS fragments generated by thorough dialysis, and analyzed the resultant material by HPLC. The profiles of HNO_2-treated CS-B and untreated CS-B are shown in Fig. (**3A**). The HNO_2 treatment selectively eliminated the GlcN peak, confirming that the CS-B was indeed contaminated with HS.

We also prepared CS-B oligosaccharides by a partial N-deacetylation reaction followed by low pH nitrous treatment of both HNO_2-treated CS-B and untreated CS-B. No GlcN-containing peak was observed in the profiles of any CS-B oligosaccharides prepared from HNO_2-treated CS-B (data not shown). However, all the oligosaccharides prepared from the untreated CS-B contained significant amounts of GlcN-oligosaccharides (Fig. **3B**). The degree of GlcN- oligosaccharide contamination was greatest in the CS-B tetrasaccharides and decreased with increased chain length, but even tetradecasaccharides (CS-B-14) had significantly greater GlcN content than the original untreated CS-B sample. It appears that HS contamination-levels were greatly amplified in the process of preparing the oligosaccharides. This may be because partially N-deacetylated CS-B, which has an N-acetylated GlcN content of 100%, is more susceptible to deaminative scission than HS and heparin, which have an N-acetylated GlcN content of 60% and 80%, respectively.

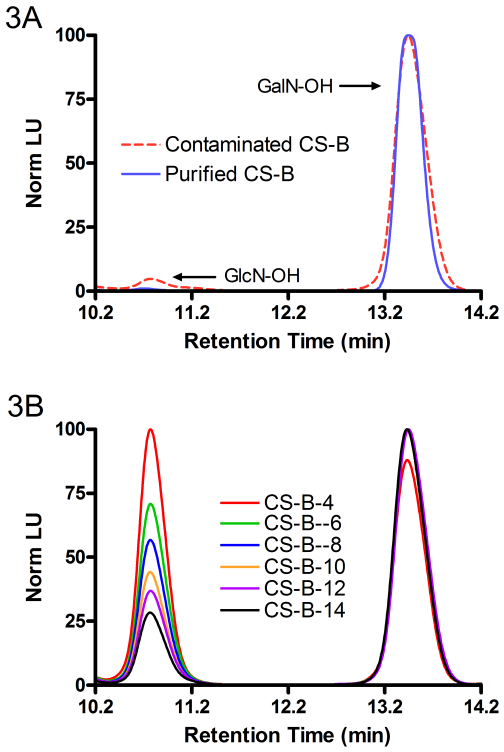

Fig. (3). (**A**) CS-B was treated with HNO_2 at pH 1.5. After thorough dialysis to remove degraded GAG fragments, the purified CS-B was quantified again by the HPLC method. (**B**) HS oligosaccharides were enriched when contaminated CS-B was used as a starting material for CS-B oligosaccharide preparations.

The Carbazole and Alcian Blue Methods

The GAGs that were analyzed by the HPLC method (Fig. **1**) were then quantified by the carbazole (Fig. **4B**) and Alcian Blue (Fig. **4A**) assays. Concentration response curves and r^2 values were determined. The r^2 values of all the GAGs tested ranged from 0.984 to 0.995 for the Alcian Blue assay and from 0.998 to 0.999 for the carbazole assay, indicating a high degree of reproducibility. However, in both assays, the concentration response curves for the different GAGs were not parallel. This suggests that, in both assays, the quantification reactions were subtly influenced by specific GAG associated properties (structure, impurity, or other factors).

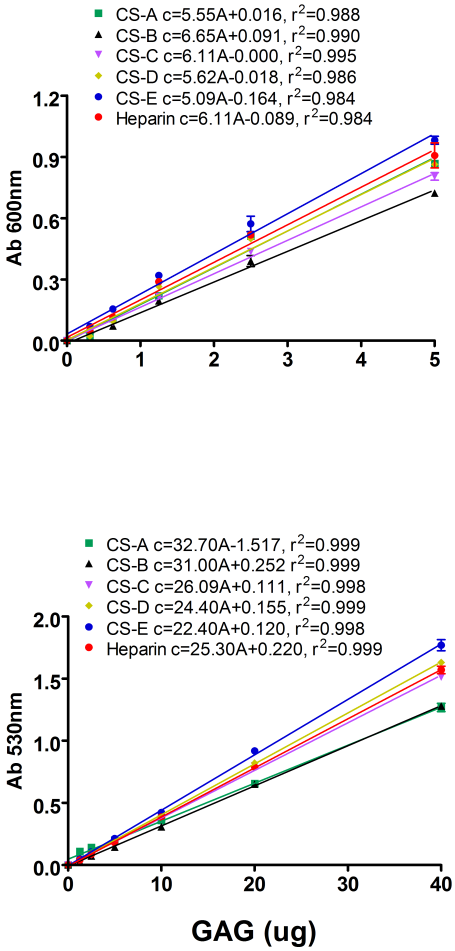

Fig. (4). The CS-A, CS-B, CS-C, CS-D, CS-E, and heparin samples examined by the HPLC method (Fig. **1**) were assayed by the Alcian Blue method (panel **A**) and the carbazole method (panel **B**). The concentration response curves, equations, and coefficient of correlation (r^2) for each GAG are representative of over ten independent experiments. Each reaction was performed in duplicate. The variable '**A**' is the measured absorbance and 'c' is equal to the quantity of GAGs (in µg).

Comparison of the Quantification by HPLC & Carbazole Methods

The carbazole assay uses GlcA as an external standard. Thus, a calibration curve can be produced for GlcA (Fig. **5**) in addition to those generated for the GAG samples (Fig.

4B). The GlcA curve fitting equation (in this case c = 26.78A + 0.4524 with an r^2 value of 0.9992 where A is measured absorbance and c is the amount of GlcA in nmol) can then be used to calculate the amount of GlcA that corresponds to 1µg for each GAG (Table **2**, column 4).

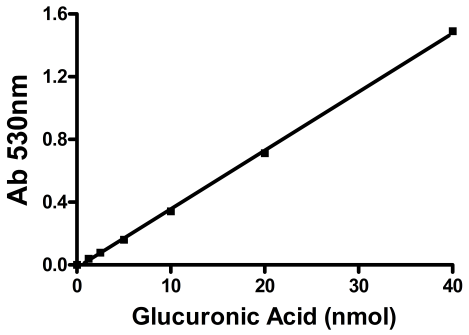

Fig. (5). A 50mM GlcA stock solution was made in water. 0, 1.25, 3.5, 5, 10, and 40nmol dilutions were prepared in duplicate to be used in the generation of an external calibration curve. The curve was used for calculating the GlcA content of the GAG samples. The data shown is representative of more than ten independent experiments.

We then compared the GAG content of each sample based on the GlcA standards (Table **2**, column 7), and the GAG content determined by HPLC (Table **2**, column 6). As seen in Table **2**, the calculated concentration of nearly all the samples was significantly less than the theoretical GAG stock concentration (1mg/ml), which was formulated based on dry weight. This indicates the presence of impurities or water in the GAG samples. Of note, in the case of CS-A, the value obtained with the carbazole assay (1.27mg/ml) is greater than its theoretical maximum (1.00mg/ml). Moreover, we also noticed that the CS-A concentration response curve (c=32.7A-1.517) differed significantly from the response curves of the other GAGs (Fig. **4B**). CS-A produced a non-linear response, producing relatively high absorbance at low concentrations and relatively low absorbance at high concentrations. These observations led us to conclude that the values yielded by the carbazole assay for the CS-A sample are inaccurate.

In the Carbazole Assay Salts Generated a False Positive

Comparison of the GAG quantification curves obtained for the carbazole and Alcian Blue assays (Fig. **4**) suggests the subtle influence of uncontrolled factors on the quantification reactions. These discrepancies could simply be due to differences in GAG purity or water content. Nevertheless, we examined the impact of salt on the two assays and observed that NaCl increased the background absorbance in the carbazole assay in a concentration-dependent manner (Fig. **6A**). We calculated that 0.01mM NaCl produced an increase in signal intensity equivalent to that generated by 4µg CS-A or Heparin. In addition, the GAG and salt signals were additive. In short, the carbazole assay had about 6-fold higher specificity for GAGs than for the NaCl. These results indicated, for example, that the quantity of GAGs dissolved in PBS (0.15M NaCl) could be overestimated by up to 60µg/ml by the carbazole method. In contrast, salt concentrations of up to 1M NaCl had minor effects on the Alcian Blue assay (Fig. **6B**) and no detectable effects on the HPLC assay (Fig. **7**).

DNA Contamination Showed no Effect on the Carbazole or Alcian Blue Assays

Biological samples, especially GAGs purified from biological sources, may contain DNA. Thus, we examined the impact of DNA contamination on the quantification assays. We compared the signal generated by 0.1mg/ml CS-A and heparin in the presence of 0 to 0.2mg/ml DNA. We measured two 10µl duplicates for each sample in the Alcian Blue assay and two 40µl duplicates for each sample in the carbazole assays. The results indicated that within this DNA concentration range, neither the Alcian Blue assay (Fig. **8A**) nor the carbazole assay (Fig. **8B**) was affected by DNA contamination.

The Alcian Blue Assay is Sulfation Dependent

Next we examined the impact of sulfation on the GAG assays. We used for this purpose three heparin species, each with a different degree of sulfation. Heparin is the most sulfated species, NDSNAc-heparin provides an intermediate degree of sulfation, and CDSNAc-heparin is the least sulfated species. The HPLC assay and the carbazole assays could detect all three different heparin species (Fig. **8D**), and concentrations were the same when measured by the carbazole (Fig. **8D**) and HPLC assays (HPLC data not shown). In contrast, the Alcian Blue assay did not detect CDSNAc-heparin, the unsulfated species (Fig. **8C**). This last result was consistent with a previous report which indicate that the Alcian Blue assay cannot detect HA, another unsulfated GAG [23]. The Alcian Blue assay did yield similar concentration response curves for sulfated GAGs ranging from 1 to 2.7 sulfates per disaccharide (Fig. **4A**). Thus, the Alcian Blue assay can be used to quantify mixtures of sulfated GAGs.

Table 2. Quantification by HPLC and Carbazole Methods

	GlcN (pmol)	GalN (pmol)	GlcN+GalN (pmol)	GlcA (pmol)	GlcN+GalN:GlcA ratio	GAG, HPLC (mg/ml)	GAG, Carbazole (mg/ml)
CS-A	201	1250	1452	2514	0.58	0.74	1.27
CS-B	117	1470	1587	1099	1.44	0.80	0.56
CS-C	148	1208	1356	1375	0.99	0.69	0.70
CS-D	0	1564	1564	1380	1.13	0.89	0.78
CS-E	0	1847	1847	1505	1.23	1.05	0.85
Heparin	954	0	954	1278	0.75	0.61	0.82

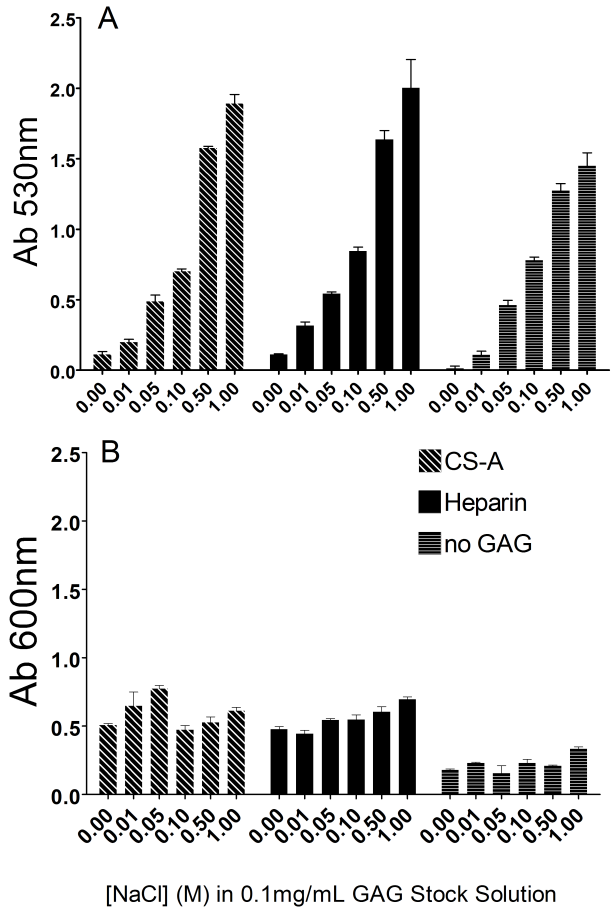

Fig. (6). CS-A (0.1mg/ml) or Heparin (0.1mg/ml) was dissolved in 0, 0.01, 0.05, 0.10, 0.5, and 1M NaCl solutions. Control solutions contained 0.01, 0.05, 0.10, 0.5, and 1M NaCl only. (**A**) 40µl duplicates of each solution were assayed by the carbazole method; (**B**) 10µl duplicates were assayed by the Alcian Blue method.

DISCUSSION

The HPLC assay is the most sensitive GAG quantification method available. It can be used to quantify the major classes of GAGs and can determine whether glucosamine GAGs, such as HS, KS, and HA, are contaminated with galactosamine GAGs, such as CS and DS or vice versa. However, the assay is time consuming and requires an HPLC with a fluorescence detector and Pico-tag hydrolysis station, instruments that are not available in most laboratories. Other methods have been developed for general carbohydrate analysis [26] and in some cases they can substitute for the HPLC method if the GAGs are completely hydrolyzed into non-sulfated uronic acid and glycosamine. This procedure also requires a Pico-tag hydrolysis station because GAGs are more difficult to hydrolyze than N- or O-linked oligosaccharides.

GAGs are relatively heterogeneous polysaccharides whose biological activities are usually determined by specific structural subsets. Thus the capacity to distinguish among GAG structural classes and detect possible GAG-cross contamination can be essential in the elucidation of

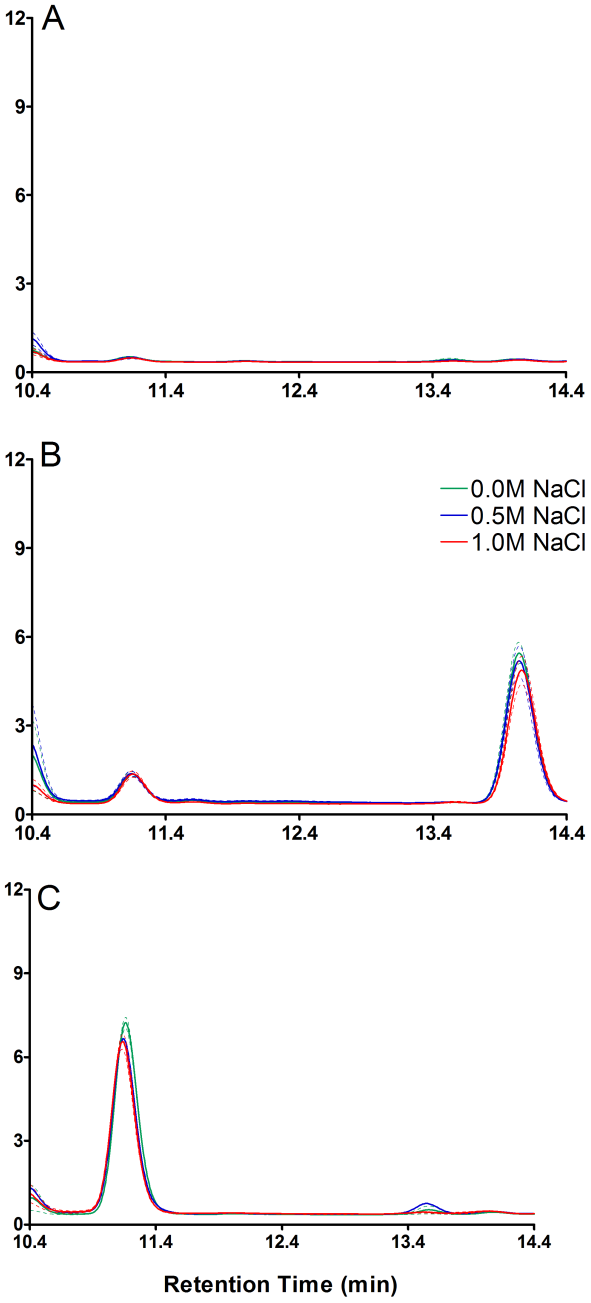

Fig. (7). CS-A (0.01mg/ml) or heparin (0.01mg/ml) was dissolved in 0, 0.5, and 1M NaCl and 1µl duplicates were independently hydrolyzed and analyzed by the HPLC method.

GAG-ligand interactions. Not all GAG classes can be reliably detected by enzymatic degradation, for example it is well established that mammalian and bacterial hyaluronidase [27] and chondroitinase [28] digest both CS and HA. Similarly, nitrous acid treatment can be used to remove heparin, but it can also generate nitrous acid resistant HS fragments that can co-purify with other GAGs. Thus, the HPLC method remains the assay of the choice to distinguish among the major GAG structural groups.

Both the carbazole and the Alcian Blue assays [23] can be performed in most laboratory settings, although neither

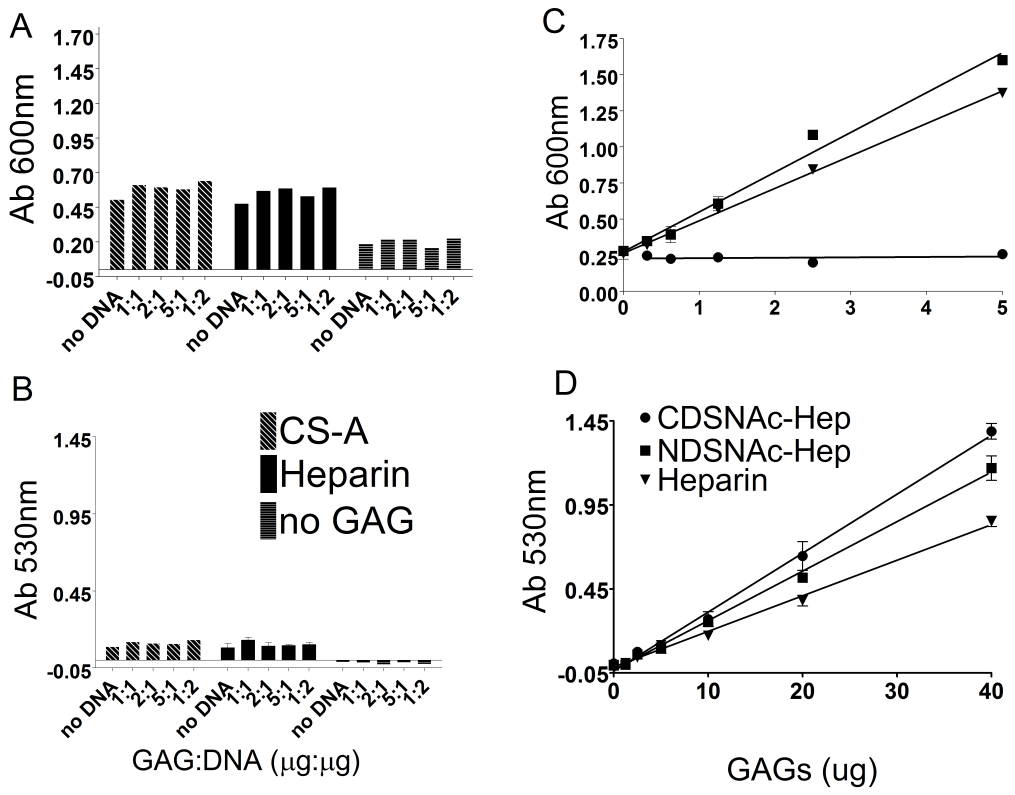

Fig. (8). CS-A (0.1mg/ml) or heparin (0.1mg/ml) was dissolved in solutions containing 0, 0.02, 0.05, 0.1, and 0.2mg/ml DNA. Two 10μl duplicates of each solution were assayed by the Alcian Blue method (**A**) and two 40μl duplicates were assayed by the carbazole method (**B**). Heparin, NDSNAc-heparin, and CDSNAc-heparin were quantified by the Alcian Blue (**C**) and carbazole (**D**) assays. The data shown is representative of three independent experiments.

can distinguish between glucosamine- and galactosamine-containing GAGs. The carbazole assay is based on the quantitative concentration-dependent color reaction between carbazole and uronic and can be as reliable as the HPLC assay for quantifying uronic acid containing GAGs [21,22]. However, the carbazole assay cannot detect KS, which lacks uronic acid residues. We also observed that NaCl contamination generated false positive signals. Thus, it is essential that GAG samples must be salt-free. We observed previously that the carbazole assay overestimates concentration for GAGs that had undergone size exclusion chromatography in a salt containing buffer (Zhang L., unpublished data). The salt effect described in this report accounts for that observation. Since gel filtration is a common way of obtaining biologically active GAG oligosaccharide fragments, removing the salts before performing the carbazole assay must be included for achieving an accurate GAG quantification. Interestingly, Bitter and Muir also reported that the carbazole assay is sensitive to glucose, and that certain types of GAG signals are sensitive to chloride ions [21]. We conclude that the validity of the carbazole assay is largely dependent on GAG purity.

The sensitivity of our carbazole assay was 5-fold higher than that of the conventional assay [21,22] due to a 5-fold reduction in the reaction volume. It can detect as little as 1.25nmol (1.25nmol x 194 = 243ng) GlcA (Fig. **4**) which produces a signal of 0.040±0.002 absorbance units above the background. In contrast, the conventional carbazole assay consumes ~ 2-5μg of GAGs. Cesaretti *et al.* also miniaturized the carbazole assay and adopted the same reaction volume that we used, but in a 96-well format [29]. Surprisingly, the sensitivity of their assay is identical to the conventional tube assay, i.e., 1μg for GlcA and 3μg for GAGs. While they theoretically should have observed the same enhanced sensitivities that we did, it appears likely that the stringent temperature control required for thorough GAG hydrolysis and carbazole reactions cannot be achieved in a 100°C oven with an open lid 96-well format [29]. Indeed, when we adapted our reactions to their 96-well platform we observed a dramatic loss in GAG sensitivity (data not shown).

The Alcian Blue assay is the fastest way to quantify GAGs. Alcian Blue is a tetravalent cationic dye with a hydrophobic core that contains copper, which gives it its Blue color. Alcian Blue interacts with sulfated GAGs with high specificity at a pH low enough to neutralize all carboxyl and phosphoric acid groups and at an ionic strength great enough to eliminate ionic interactions other than those between Alcian Blue and sulfated GAGs. Based on the specific interactions between sulfated GAGs and Alcian Blue, the GAG quantification assay, in addition to other applications, has been developed [23].

The Alcian Blue protocol we used is 4-fold more sensitive than the miniaturized carbazole assay (Fig. **3**) and the dot blot/reflection Alcian Blue assay [30] is even more sensitive. However, there are multiple issues associated with the Alcian Blue assay. First, Alcian Blue is not a well-defined compound; the solubility and GAG-binding characteristics of Alcian Blue not only differ among different manufacturers but also vary between batches from the same manufacturer.

Thus, preliminary experiments are required to characterize the specific Alcian Blue dye before using it for quantification. Second, Alcian Blue-GAG interactions are sulfation and structure dependent; Alcian Blue does not bind non-sulfated GAGs [31]. Third, since this assay only detects sulfated polysaccharides, there is no monosaccharide or oligosaccharide "standard" GAG that can be used in the Alcian Blue assay to generate concentration response curves for GAG quantification. Therefore, the Alcian Blue assay is a "relative" GAG quantification method by nature.

In summary, the HPLC method should be the primary choice for quantification of both commercially available GAGs and GAGs purified from biological sources because it is extremely sensitive and it reliably distinguishes glucosamine- and galactosamine-containing GAGs. The carbazole assay is as reliable as the HPLC assay for the quantification of uronic acid containing GAGs, but the GAGs must be free of salts and other sugars such as glucose. The Alcian Blue assay is a fast and easy way to quantify GAGs but the values obtained by this assay should be interpreted with care because of its dependence on sulfation.

Multiple proteoglycans, such as syndecan, endocan, and glypican, and GAG structure editing enzymes, such as heparanase, sulfatases, and hyaluronidases, have been alleged to be cancer diagnosis and prognosis markers, or treatment targets [32-39]. The biological functions of proteoglycans are largely due their GAG chains. Thus, simple, sensitive, and robust GAG quantification methods are needed for the development of biomarkers. We suggest that an automated HPLC assay would be generally useful for the routine measurement of a panel of GAG-based biomarkers while the carbazole assay and the Alcian Blue assays could prove valuable for more specific purposes.

MATERIALS & METHODS

GAG Sources and Storage

Heparin from porcine intestinal mucosa (H4784), CS-A from bovine trachea (C8529), CS-B from porcine intestinal mucosa (C3788), and CS-C from shark cartilage (C4384) were purchased from Sigma. CS-D rom Shark cartilage (Cat. # 230689) was purchased from Calbiochem. Two 1µg samples of squid cartilage CS-E were purchased, one from Calbiochem (cat. # 230690) and one from Seikagaku (Cat. # 400678). A chemically modified heparin kit with completely desulfated, re-N-acetylated (CDSNAc) and N-sulfated, re-N-acetylated (NDSNAc) heparin was purchased from Seikagaku (Cat. # 400645). HPLC analysis revealed that the NDSNAc-heparin had ~3.8% GalN-containing GAG contaminations whereas no GalN was observed in the CDSNAc-heparin. These results were in agreement with our early observations that GAG-cross contamination was batch-dependent (Figs. **1** and **2**).

We observed that GAG concentration gradually decreased when GAGs were stored at 4°C at a concentration of 1mg/ml. We made 10mg/ml GAG aliquots in siliconized tubes and stored them at -20°C. For quantification purposes, we made fresh 1mg/ml of GAG working solutions from the 10mg/ml stock solution. For the carbazole and Alcian Blue assays, we made 200µl master serial dilution solutions in water containing 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125 mg/ml, 0.0625mg/ml, or 0.03125mg/ml GAGs. Two 10µl duplicates of each GAG dilution were used for the Alcian Blue assay (10µg, 5µg, 2.5µg, 1.25µg, 0.625µg, and 0.3125 µg, respectively) and two 40µl duplicates were used for the carbazole assay (40µg, 20µg, 10µg, 5µg, 2.5µg, and 1.25µg, respectively).

The HPLC Method

GAGs were subjected to acid hydrolysis, sodiumborohydride reduction, precolumn derivatization with o-phthaldialdehyde (OPA) and 3-mercaptopropionic acid (3MPA), and reversed phase HPLC separation with fluorescence detection of the isoindole derivatives. GAG aliquots containing 360pmol of norleucine as an internal standard were dried in pyrolized glass vials (Agilent, Palo Alto, CA, part 5181-8872) before hydrolysis with HCl vapor in N_2 gas at 100 ºC for 3 hours. The samples were rehydrated in 45µl of 0.56% $NaBH_4$ to reduce the glucosamine and galactosamine liberated by acid hydrolysis into glucosaminitol and galactosaminitol, respectively. After an overnight incubation at room temperature, the reaction was terminated by adding 5µl of 2N acetic acid to each vial. The sample was dried by speed-vac and dissolved in 5µl water for precolumn derivatization with 35µl of 7.5mM OPA, 375mM 3MPA, in 0.4N borate adjusted to pH 9.3 with NaOH. Half of this reaction mixture was injected onto a 4.6 x 250mm C-12 column, a Synergi 4µ MAX-RP 80 Å (Phenomenex, Torrance, CA, part 00G-4337-E0), and heated to 35 ºC. The column was equilibrated with Buffer A, consisting of 0.05M (monobasic and dibasic) sodium phosphate, pH 7.2 in 25% methanol, at a flow rate of 0.8ml/min. Buffer B consisted of methanol, water, and tetrahydrofuran at 70:30:3 volume ratios. After injection, Buffer B was increased from 0 to 8% by a linear gradient between 0 and 3 min, was maintained at 8% between 3 and 18 min, at 55% between 18 and 30.5 min, at 100% between 30.5 and 32.5 min, and at 0 % between 32.5 and 35 min. A 5 min post-run interval at 0% B preceded the initiation of the next precolumn derivatization injection sequence. The fluorescent derivatives of glucosaminitol, galactosaminitol, and the amino acids contained in the GAG preparations were excited at 337 nm and detected at 454 nm. A more detailed description of this assay can be found in the original publication [20].

The Carbazole Method

Carbazole-based quantification involves two steps: 1.) hydrolyzing GAGs into uronic acid and glycosamine with 0.025M sodium tetraborate (Sigma) in sulfuric acid; and 2.) coloring uronic acid with carbazole (Sigma). Both reactions require boiling for 10 min. A glass tube containing 3.6ml of reactants with a marble on top is usually used for the carbazole assay. The marble prevents splashing of sulfuric acid during boiling and the potential hazard caused by the tube's cap popping. The assay sensitivity is 1µg for GlcA and 3µg for GAGs. For GAG quantification, at least two ~5µg duplicate samples of unknown the GAG are required. We reasoned that if we scaled down the reaction volume, the same amount of GAGs would generate more concentrated colors, and thus enhance the assay sensitivity of this assay.

We used a PCR instrument set at 100°C for the hydrolysis and carbazole reactions. The reagents used in the carba-

zole assay were 0.025M sodium tetraborate·10 H_2O in sulfuric acid (H_2SO_4+Borex) and 0.125% carbazole in absolute ethanol (W/V). We added 200µl of the H_2SO_4+Borex solution to 40µl of GAG solution or water (blank control). The tubes were capped and vortexed briefly. The tubes were then put into a PCR instrument with the lid securely locked. The PCR instrument was programmed to run for 15 min at 100°C and then cool down to 4°C. To each tube, 8µl of carbazole solution was added followed by a brief vortex. The tubes were returned to the PCR instrument for the carbazole reaction, which was run at 15 min at 100°C and then cooled to 4°C. The samples were vortexed and 200µl of each sample was transferred to a 96 well plate. The absorbance at 530nm was measured by a Spectra MAX M2 plate-reading spectrophotometer (Molecular Devices, Sunnyvale, CA). GlcA was used as an external standard, and the standard curve was derived by plotting absorbance against the concentration of GlcA or standard GAG.

The Alcian Blue Method

We used a simpler Alcian Blue assay [24] than the assay described by Karlsson and Bjornsson [23]. In brief, the Alcian Blue dye stock solution consisted of a 1/100 dilution of the original dye solution (1g Fluka Alcian Blue 8GS with 100ml of 18mM H_2SO_4). The 1/100 dilution was brought to an absorbance of ~1.4 at 600nm by adding additional dye (if needed). This was followed by centrifugation at 10,000rpm for 30 min to remove insoluble dye particles. The working dye solution containing 0.25% Triton X-100, 0.018M H_2SO_4 and 10% dye stock was made and filtered (0.2µm filter). 10µl of a solution containing 0.027M H_2SO_4, 0.375% Triton X-100, and 4M guanidine HCl was added to each 10µl aliquot of a GAG standard series (0, 0.3175µg, 0.625µg, 1.25µg, 2.5µg, 5µg, and 10µg in 10µl of water) along with 100µl of working dye solution. After microcentrifugation for 10 min at 16000*g* at 4°C, the supernatant was aspirated and the pellet was dissolved in 500µl of 8M guanidine HCl by rigorous vortexing.

The samples were centrifuged for 3 min at 16000g. 300µl of supernatant was transferred to a 96-well plate. The absorbance at 600 nm was measured using a Spectra MAX M2 plate-reading spectrophotometer.

Determining Concentration Response Curves

The concentration response curves for both the carbazole and the Alcian Blue assays were plotted using the GraphPad Prism software. The software automatically formulated the equations, in which A is equal to measured absorbance and c is equal to the amount of GAGs expressed in µg. The base line absorbance readings and equations changed from day to day due to changes in reagents, sample preparations, or other unknown factors, but the r^2 values of both assays were always ≥ 0.98, indicating their reliability.

Comparison of GAG Quantity as Determined by the HPLC And Carbazole Assays

We calculated the A value for 1µg of each GAG by using the corresponding carbazole curve fitting equation shown in Fig. (**4B**). We then used the A values in the GlcA equation, $c = 26.78A + 0.4524$, to calculate equivalent GlcA quantities based on activity. The HPLC method includes an internal standard, norleucine (NorL), and a series of external GlcN-OH and GalN-OH standards. Based on the calibration curves of the GlcN-OH and GalN-OH, the nmols of GlcN and GalN equivalent to the fluorescence units generated by 1µg (dry weight) of GAGs were calculated (Table **2**). The 1µg GAG equivalent pmols of GlcA and the ratio of GlcN+GalN (as quantified by HPLC) to GlcA for each GAG was summarized in Table **2**. The GlcA values plotted in Fig. (**4B**) were 2524, 906, and 861pmol for 1, 10, and 40µg of CS-A respectively. 2524pmol value was used to calculate the absorbance (A) value.

Molecular Weights Of GAG Dissacharides

An analysis of GAG disaccharides by capillary HPLC/MS (not shown) indicated that CS-A, CS-B, and CS-C had ~1 sulfate per repeating disaccharide; CS-D and CS-E had ~1.6 sulfates per disaccharides; and heparin had 2.7 sulfates per disaccharide. Because all of the GAGs are in the sodium form, the mg/ml GAG concentrations were calculated based on F.W. 505 (379 for repeating disaccharides + 80 for sulfate + 2 x 23 for sodium) per disaccharide for CS-A, CS-B, and CS-C; 566.8 (379 + 1.6 x 80 + 2.6 x 23) per disaccharide for CS-D and CS-E, and 638 (337 + 2.7 x 80 + 3.7 x 23) per disaccharide for heparin.

Low pH Nitrous Treatment of HS-Containing CS-B

A standard low pH nitrous treatment procedure was used to remove HS from the contaminated CS-B [25]. In brief, CS-B was treated with HNO_2 at pH 1.5 for 30 minutes on ice. The treated CS-B sample was dialyzed against water in 5K cut-off dialysis tubing for two days and then dried and re-dissolved in water.

Preparation of Oligosaccharides from CS-B

The CS-B oligosaccharides were prepared by a previously published procedure [40]. In brief, both purified and contaminated samples of CS-B were partially deacetylated with hydrazine/hydrazine sulfate and then reacted with high pH nitrous acid, which quantitatively cleaves the glycosidic linkages following N-unsubstituted D-galactosamine residues (N-acetyl-D-glycosamine residues are resistant to deaminative cleavage at high pH). This procedure yields oligosaccharide mixtures.

The mixtures were then separated into different oligosaccharides by gel filtration on a Bio-Gel P-10 column. Aliquots of purified oligosaccharides were then quantified by the HPLC method.

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